DONOR–HOST CELL INTERACTION IN HOMOLOGOUS SPLENOMEGALY IN THE CHICK EMBRYO

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The initial step in the splenomegaly observed in the chick embryo following the implantation of a piece of adult chicken spleen on the chorioallantoic membrane (CAM) involves a migration of donor cells into the spleen and other organs of the host, as was first inferred from the serial transfer studies by Simonsen (1957), and Ebert (1957). When either the blood or cells of the enlarged spleen from the first host are injected into or grafted to a new host, a similar enlargement of the spleen in the secondary host is obtained.

Direct evidence of this migration and subsequent colonization of donor cells in the host spleen was provided by Biggs and Payne (1959). Four days after the intravenous injection of cockerel blood into 14-day-old chick embryos, they were able to identify male cells in the enlarged spleens of female embryos. More recently, Becker *et al.* (1963) were also able to demonstrate the presence of donor cells containing a distinctive chromosome marker (T6) in large white clusters of cells or colonies in the spleens of heavily irradiated (900–1000 rads) mice. These macroscopic colonies appear to be derived exclusively from the proliferative activity of "stem" cells found in mouse hematopoietic tissues, as well as fetal liver. When highly inbred strains of mice were used, an "inhibition" rather than a stimulation of response was obtained, which was difficult to explain as either an immunologic "graft *vs.* host" or "host *vs.* graft" reaction (McCulloch and Till, 1963). In the chick, on the other hand, the resulting host spleen enlargement appears to be mediated by the migration, colonization, and extensive immunologic donor-host cell interaction in the host spleen.

In autoradiographic studies using tritiated thymidine, although distinctly labeled cells were found in the adult spleen graft on the CAM, few labeled cells were detected in the host spleen (Mun *et al.*, 1962). This observation argues against a massive migration of donor cells into the host spleen.

The rate of migration of these donor cells appears to be rapid. Mun *et al.* (1962) observed that as early as two days after a piece of adult chicken spleen was implanted, when the host spleen was then transferred to the CAM of a new host, although no enlargement of the embryonic spleen was detectable, it was able to elicit splenomegaly in the new hosts. Grafts of chick embryo spleen implanted approximately 1 cm. away from the adult spleen graft, when transferred to the CAM of new hosts, were also able to stimulate growth.

Within the host spleen, some of the invading adult cells proceed to launch an extensive "immunologic" attack. The immunological basis of this reaction has been

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well established. Danchakoff (1918) noted that embryonic spleen tissue was not able to stimulate growth. This ability was gradually developed in hatched chicks from the fifth day to the third week (Solomon, 1961; Mun *et al.*, 1962). Studies using highly inbred lines of chickens support the hypothesis that the splenic enlargement is due in part to the reaction of adult donor cells against the foreign antigens encountered in the host (see reviews of Billingham, 1959, and Ebert and DeLanney, 1960).

The steps immediately following this immunologic attack are not quite as clear. Do the invading donor cells, having been stimulated by the host antigens, proceed to proliferate and form antibodies and eventually destroy or replace the native cell population (Simonsen, p. 449, 1957)? Or, do the donor cells, without undergoing any further proliferation, stimulate the *host* cells to divide? Or, both?

In the same study described above, Biggs and Payne (1959) found that, of the 170 cells examined in the enlarged spleen of female embryos, 75 were male cells and 95 were female cells. Thus, an appreciable portion of the splenic enlargement was provided by the host. Because a ratio of approximately 1:1 was observed in spleens enlarged five- to twenty-fold, this observation suggests not only that an appreciable portion of the splenic enlargement is provided by the host, but also that considerable proliferation of both donor and embryonic host cells is involved.

DeLanney *et al.* (1962) also observed a host response in the adult spleen graft on the chorioallantoic membrane. Cytological studies of the graft, at one- to two-day intervals, from the 1st to 10th day post-implantation, revealed dense aggregations of hemocytoblasts in the adjacent membranes by the 4th day post-implantation. Granuloeytes, usually associated with an immunological type of response, were found in significant concentrations by the 5th day post-implantation.

In order to analyze the mechanism of stimulation of growth in the embryo, we may first inquire: Is the proliferation of embryonic cells necessary to produce the observed enlargement? Or is the proliferation of adult donor cells sufficient? What is the role of the adult donor cells in this stimulation? Is active proliferation of donor cells necessary to bring about the stimulation of embryonic components? The main objective of this study is to elucidate the role of the embryonic or host component in this splenomegaly reaction. We shall first examine the sufficiency and/or necessity of embryonic cells in this phenomenon and then explore the mechanism of this stimulation to proliferation (Mun, 1963).

Materials and Methods

The chorioallantoic membrane grafting technique (Willier, 1924; Hamburger, 1960) was used throughout this study. A quadrilateral window 1×1 cm. was cut in the shell with a fine-toothed hacksaw blade (Milford Midget, HS32T $\frac{1}{4} \times 0.014$ inch without set, Henry G. Thompson & Son Company, New Haven, Connecticut). A piece of tissue approximately $1 \times 1 \times 2$ mm. and weighing 5 to 10 mg. was placed on the exposed membrane. The shell membrane and shell were replaced and sealed with paraffin. The eggs were returned to the incubator with the pointed end down.

5-Fluorouracil (5FU) was provided by Hoffmann-La Roche Laboratories, Nutley, N. J. Approximately 0.35 to 0.40 mg. of 5FU in 0.07- to 0.08-ml. volumes were injected into a blood vessel in the chorioallantoic membrane in the direction of

flow (Terasaki and Cannon, 1957). A one-ml. syringe and a 30-gauge needle with a 22° bend was used. The syringe was held in place with a thermometer clamp and the egg was moved into position by means of a Cenco-Lerner Micro Labjack (Central Scientific Co., Chicago, Ill.).

The host spleens were removed on the 17th or 18th day of incubation and weighed to the nearest 0.2 mg. The graft on the CAM was graded according to the degree of incorporation and vascularization (Mun, Kosin and Sato, 1959). The embryo was drained, blotted on paper, and weighed to the nearest 0.1 gram.

I. The Role of the Host Component in the Splenomegaly Reaction

To determine whether embryonic or host cell proliferation plays a sufficient and/or necessary role in this splenomegaly reaction, two experiments were conducted in which two different strains of chickens were used: (1) a highly inbred line (Line 7, Regional Poultry Research Laboratory, East Lansing, Michigan) with a coefficient of inbreeding of greater than 95%, and (2) a commercial strain (Queens Line, Arbor Acres Hatchery, Skowhegan, Maine).

Table I

The effect of adult spleens from Line 7 chickens (A7) on the mean spleen weight of Line 7 (E7) and Queens Line (QL) host embryos

Adult donor	First host embryo		Second host embryo		
Line	Line	Mean spleen wt. (mg. ± S.D.)	Line	Mean spleen wt. (mg. ± S.D.)	Group
A7	E7	8.91 ± 4.66 (18)	E7	10.80 ± 1.55 (15)	1
			QL	24.57 ± 20.10 (25)*	2
	QL	68.41 ± 33.18 (19)	QL	70.64 ± 36.16 (33)**	3
			E7	18.32 ± 9.74 (29)**	4
Chick Ringer saline	QL	8.72 ± 1.55 (9)	QL	10.28 ± 4.20 (8)	5
Chick Ringer saline	E7	8.76 ± 1.30 (8)	E7	11.33 ± 0.88 (6)	6

*($p<0.05$).
**($p<0.01$).
Figures in parentheses indicate number of cases.

A piece of adult spleen from an adult Line 7 chicken was implanted on the CAM of 10-day-old Line 7 and Queens Line embryos. After 7 more days of incubation, the host spleens were removed, weighed, then cut in half or in 5 mg. pieces, and transferred to the CAM of new 10-day-old Line 7 and Queens Line hosts. After seven more days of incubation, the spleens from the second hosts were removed and weighed. Thus four groups or series were established.

As may be seen in Table I, when the 1st and 2nd host embryos were derived from the same line as the adult donor (Line 7), the host spleens did not increase in size

(Group 1). However, when the second host was derived from a line different from that of both the adult donor or the first host, we obtained an increase in the weight of the host spleen (Group 2). Because donor spleen tissues from either 17-day-old Line 7 (Group 6) or Queens Line embryos (Group 5) were not able to stimulate growth, we may suggest that a sufficient number of competent adult Line 7 cells was probably transferred from the first host to the second host. These competent cells then proceeded to bring about the splenic enlargement observed in the second host (Group 2).

We may also note that, although an enlargement of the second host spleen was obtained, the spleen of the first host was not enlarged in Group 2. This observation suggests that the number of cells involved in the "graft *vs.* host" reaction is not large, which agrees with previous observations in which we failed to detect a large number of H^3 thymidine-labeled donor cells in the host spleen (Mun *et al.*, 1962).

In the third group, when both the first and second hosts were derived from a line different from that of the adult donor, both hosts' spleens were enlarged. However, in the fourth group, although the second host was derived from the same line as the adult donor, we also obtained a significant ($P < 0.01$) increase in size of the host spleen, suggesting that both adult donor *and* embryo cells proliferate in the first host and are transferred to the second host in sufficient quantities to elicit splenomegaly.

Whether the proliferation of host cells was necessary in this phenomenon may be ascertained by exploring the consequences of their inactivation or elimination. Mun, Kosin and Sato (1959) have previously studied the effects of inactivation of adult chicken spleen tissue by x-irradiation. With increasing dosage of x-irradiation the ability of the donor tissue to elicit splenomegaly was gradually diminished. Heating or freezing the adult spleen tissue before implantation on the CAM also removed its ability to stimulate growth. This suggested that viable adult cells capable of undergoing mitosis were probably necessary to elicit growth.

In an attempt to inactivate the embryonic component, 5-fluorouracil (5FU) was injected intravenously into chick embryos following the implantation of adult chicken spleen on the chorioallantoic membrane.

II. The Effect of 5-Fluorouracil on the Splenomegaly Reaction [2]

5-Fluorouracil has been found to be a highly effective inhibitor in many rapidly growing systems, including regeneration of the liver after partial hepatectomy, growth of the seminal vesicle induced by testosterone, growth of epiphyseal cartilage induced by growth hormone, and foetal rat growth (Paschkis *et al.*, 1959). It also inhibits the growth of mouse and rat tumors (Heidelberger *et al.*, 1957a, 1957b, 1958; Law, 1958), Rous sarcoma (Bather, 1960) and many human carcinomas (Ansfield and Curreri, 1959; Ansfield, Schroeder and Curreri, 1962). Karnofsky, Murphy and Lacon (1958) injected 5FU into the yolk sac of 8-day-old chick embryos and observed feather inhibition and edema. An LD_{50} of 1.0 mg. 5FU/egg at 8 days of incubation was established.

[2] From work reported in a thesis by Mr. E. R. Burns, submitted to the Faculty of the Graduate School in partial fulfillment of requirements for the degree, Master of Science, in the Department of Zoology, University of Maine, June, 1963.

To determine the effect of 5FU on the splenomegaly phenomenon, 0.25 mg. to 0.75 mg. of 5FU was injected intravenously into 11½-day-old chick embryos which had previously received chorioallantoic membrane grafts of adult chicken spleen on the tenth day of incubation. The 1½-day interval between graft and injection was judged to be adequate to permit a sufficient number of donor cells to migrate to the host spleen (Mun *et al.*, 1962).

When the host spleen was recovered on the 17th day of incubation, a dose of 0.50 mg. 5FU was found to be sufficient to inhibit the splenomegaly reaction (Table II). The mean spleen weights of the 0.50-mg. and 0.75-mg. 5FU groups were statistically different from the control groups receiving uracil, but not different from

TABLE II

The effects of 5-fluorouracil (5FU) 1½ and 3½ days after the implantation of adult spleen

Number of days after implantation	Treatment	No. cases	Mean spleen wt. (mg. ± S. D.)	Mean body wt. (mg. ± S.D.)
1½	0.25 mg. 5 FU	12	62.20 ± 42.14	14.24 ± 1.85
	0.25 mg. uracil	4	81.85 ± 48.16	14.70 ± 2.60
	0.50 mg. 5FU	23	11.04 ± 9.48	11.65 ± 1.78
	0.50 mg. uracil	9	32.38 ± 16.76**	13.98 ± 1.50
	0.75 mg. 5FU	54	6.66 ± 4.35	10.19 ± 1.00
	0.75 mg. uracil	18	30.44 ± 15.50**	13.51 ± 2.16*
	0.1 cc. chick Ringer	13	8.52 ± 1.25	16.49 ± 1.64
3½	0.25 mg. 5FU	10	34.88 ± 11.60	13.17 ± 1.18
	0.50 mg. 5FU	7	24.82 ± 25.56	13.52 ± 1.69
	0.75 mg. 5FU	42	11.04 ± 4.66	13.71 ± 1.86
	0.75 mg. uracil	29	46.91 ± 19.04**	15.08 ± 1.55
	1.50 mg. 5FU	10	5.53 ± 1.34	13.20 ± 1.20
	0.1 cc. chick Ringer	7	10.57 ± 2.32	16.94 ± 1.42

*($p < 0.05$).
**($P < 0.01$).

the mean spleen weight of sham operated embryos. In severely inhibited embryos, feather inhibition, edema, and other anomalies were observed.

When 5FU was injected into 13½-day-old chick embryos, 3½ days after the implantation of adult chicken spleen, 0.75 mg. 5FU was found to be sufficient to inhibit the splenomegaly. However, the teratogenic effect of the drug was greatly reduced or absent.

This inhibition of the splenomegaly reaction may be due to the action of the drug on the donor or host cells, or both. However, when the inhibited or unenlarged host spleen was transferred to the CAM of a new host, it was able to elicit splenomegaly (Table III). This observation revealed that although proliferation

of the donor cells was inhibited, viable and competent adult donor cells were still present in the host spleen.

III. The Stimulation of Embryonic or Host Component in the Splenomegaly Reaction

We now ask: why was a stimulation of growth not obtained in the spleen of the first host? Was it due to the inhibitory action of the drug on the proliferation of the adult cells? Or was it due to the blocking action of 5FU on the proliferation of embryonic host cells following stimulation by the adult donor cells? Or both? If the ratio of donor to host cells in the enlarged spleen is approximately 1:1, as observed by Biggs and Payne (1959), then there is probably some inhibition of the mitotic activity of the adult donor cells. This block may have been removed upon transfer of the host spleen to the CAM of a new host. However, are these inhibited donor cells in the host spleen capable of stimulating embryonic cells?

Table III

The effect of the transfer of embryonic and adult spleen grafts to new embryonic hosts

Days after implantation	First host			Second host	
	Treatment	No. cases	Mean spleen wt. (mg. ± S.D.)	No. cases	Mean spleen wt. (mg. ± S.D.)
$1\frac{1}{2}$	0.75 mg. 5FU	17	6.67 ± 4.31	14 spleens	28.24 ± 13.76
	0.75 mg. uracil	3	21.26 ± 5.38	3 spleens	40.27 ± 13.85
	0.1 cc. chick Ringer	6	9.82 ± 1.44	3 spleens	8.90 ± 0.60
$3\frac{1}{2}$	0.75 mg. 5FU	9	10.28 ± 2.24	12 grafts	52.93 ± 34.73
				9 spleens	48.97 ± 25.63
	0.75 mg. uracil	12	34.02 ± 15.40	7 grafts	55.25 ± 35.37
				4 spleens	95.05 ± 51.16
	0.1 cc. chick Ringer	2	11.44 ± 2.27	3 spleens	10.26 ± 0.81

To examine this possibility, as well as to establish the role of the embryonic component in this splenomegaly reaction, fresh embryonic spleen tissue was added to the same host which had previously received a chorioallantoic graft of adult chicken spleen and an intravenous injection of 5-fluorouracil. If embryonic cells are involved in this splenomegaly reaction, then a definite response to the second embryonic spleen graft should ensue, even though active proliferation of the donor component was curtailed.

The following experimental procedure was adopted:

A piece of adult chicken spleen was implanted on the CAM on the 9th day of incubation. Two days later, on the 11th day of incubation, 0.35 mg./0.07 ml. of 5FU was injected intravenously. Three days later on the 14th day of incubation, a piece of 17-day-old chick embryo spleen was implanted on the CAM approximately 1 cm. away from the first graft.

On the 18th day of incubation, the shell was removed, together with the adhering

chorioallantoic membrane in which both the first and second grafts were imbedded. The lengths and widths of the two grafts were measured with a pair of vernier calipers to the nearest 0.1 mm. The grafts were then cut out, trimmed of all excess membranes and weighed to the nearest 0.2 mg. The host spleen was removed and weighed to the nearest 0.2 mg. The embryos were separated into 4 groups according to the degree of abnormality induced:

Group I. Embryos appeared normal.

Group II. Body weight appeared normal but feathers were approximately one-half of normal length, giving embryos a fuzzy appearance.

Group III. Body weight was more than 3 grams below normal. Feather formation was inhibited by at least two stages (Hamburger and Hamilton, 1951).

TABLE IV

Effect of adult spleen (AS) and/or embryonic spleen (ESG_1) and 5-fluorouracil (5FU) on a second embryonic spleen graft (ESG_2)

Treatment			Mean host spleen wt. (mg. ± S.D.)	Mean ESG_2 wt. (mg. ± S.D.)
Day of incubation				
9-day	11-day	14-day		
AS + ESG_1	—	—	50.02 ± 18.99 (9)	
AS	5FU	—	16.50 ± 9.85 (10)	
AS + ESG_1	5FU	—	13.51 ± 4.22 (7)	
AS	5FU	ESG_2	9.85 ± 4.68 (28)*	34.71 ± 13.33 (28)**
ESG_1	5FU	ESG_2	6.96 ± 2.69 (16)	17.83 ± 7.44 (16)
AS	—	ESG_2	29.66 ± 15.17 (18)**	33.60 ± 14.50 (18)**
ESG_1	—	ESG_2	12.45 ± 2.89 (17)	20.55 ± 8.74 (16)
—	5FU	AS	6.73 ± 3.69 (24)	
—	—	AS	20.36 ± 22.12 (18)	

*($p < 0.05$).
**($P < 0.01$).
Figures in parentheses indicate number of cases.

Group IV. Body weight was more than 3 grams below normal. Feather formation was inhibited more than two stages. Other abnormalities were also present, including edema in the head and/or abdominal regions, twisted beaks, and curled toes.

Only embryos from Groups III and IV were included in the tabulations.

As may be seen in Table IV, an enlargement of the spleen was not obtained by adding a second embryonic spleen graft into hosts which had previously received an adult spleen graft and an injection of 5FU. However, the weight of the second embryonic spleen graft was found to be significantly greater ($P < 0.01$) than that implanted in an embryo which had previously received an embryonic spleen graft and an injection of 5FU. This observation reveals not only that viable and competent donor cells are still present in the adult spleen graft, but also that these cells are capable of migrating to the second embryonic spleen graft and stimulating growth there.

Discussion

When the enlarged host spleen was transferred to the chorioallantoic membrane of a new host, it was able to elicit splenomegaly if the genetic strain of the second host was similarly different from that of the adult donor (Group 3, Table I). This suggests that the adult donor cells are capable of migrating to and invading the spleen of the embryonic hosts. Such a mechanism was inferred earlier in serial transfer studies of Simonsen (1957) and Ebert (1957), using non-inbred lines of chickens. On the other hand, when the second host was derived from the same inbred line as that of the adult donor but different from that of the first host, we also observed a stimulation (Group 4, Table I). This observation strongly suggests that embryonic spleen cells are also capable of being transferred to the spleen of the new host. Such a mechanism was inferred in a previous study in which embryonic spleen tissue was serially propagated in non-inbred embryos. After five or six transfers, a cumulative response was obtained, approximately paralleling the normal development in the chicken of the ability to elicit splenomegaly (Mun *et al.*, 1962). Because the embryonic host cells could only be transferred if they were present in adequate numbers, this observation may also lend support to the cytological observations of Biggs and Payne (1961), which indicated that both adult and embryonic host cells proliferated in the first host.

The splenomegaly reaction was clearly inhibited by 5-fluorouracil. Preliminary studies of histological sections of the inhibited spleen revealed a variety of chromosomal aberrations, suggesting that the focus of attack of the drug is upon the mitotic apparatus. Berger and Witkus (1962) found chromosomal elimination, binucleate cells and chromosomal fragmentation in *Allium cepa* root tips treated with 5-fluorouracil.

The effect of the drug on feather development was very striking. However, growth of the entire embryo appears to be affected, as judged by the stage of development (Hamburger and Hamilton, 1951) attained. When the drug was introduced on the 13½ to 14th day of incubation, its effect on growth as well as feather formation was greatly reduced or absent.

The resulting splenomegaly observed following the transfer of the 5FU-inhibited host spleens to new hosts suggests, however, that the action of the drug is not primarily on the ability of the adult donor cells to stimulate growth, but on the ability of either the adult donor cells or embryonic host cells, or both, to proliferate.

The second possibility was tested by the addition of fresh embryonic tissue to the 5FU-inhibited embryo. Because an enlargement of this second embryonic spleen graft was obtained, we may postulate that splenomegaly is due in part to the proliferation of host embryo cells following stimulation by the adult donor cells. Of course, we must assume that (1) competent adult cells in the initial graft are capable of migrating on the 14th day of incubation to the host spleen as well as to the second embryonic spleen graft in embryos injected with 5FU, and that (2) the transferred donor cells are then able to proliferate freely at either site.

Although we cannot exactly ascertain the mobility or activity of the adult donor cells on the 14th day of incubation, we can demonstrate that viable donor cells are probably already present in the host spleen because the reaction can still be transferred.

The size of the grafts on the chorioallantoic membrane may be influenced by the activity of the host cells. Tardent, Mun and Ebert (*cf.* Mun *et al.*, 1962) found labeled cells in the midst of an unlabeled adult chicken spleen graft on the CAM of a host previously injected with H^3 thymidine. Although cytological studies are not complete, gross examinations of second embryonic spleen grafts in hosts which received initially either embryo or adult spleen grafts reveal no edema or extreme thickening of the CAM.

The failure to obtain enlargement of the host spleen in 5FU-treated embryos following the addition of a second embryonic spleen graft on the 14th day of incubation is more difficult to explain, especially in the light of the first series of experiments which suggest that cells from enlarged embryonic spleens may be transferred to the spleen of the new host. Several possible explanations may be advanced. The first possibility is that the concentration of 5FU in the host spleen was still high enough to inhibit the activity of the transferred embryonic cells. This possibility, however, requires that the drug be maintained in higher concentrations in the host spleen than in the extra-embryonic membrane, or in other organs in the body, which may be unlikely. A second possible explanation is the failure of a sufficient number of embryonic cells to be transferred to the host spleen. As shown in Table IV, a striking enlargement of the host spleen is obtained when adult chicken spleen is implanted on the 10th day of incubation. However, when adult spleen was grafted on the 14th day of incubation, the stimulation was greatly reduced. This observation is in line with that of Solomon and Tucker (1963) who obtained splenomegaly when the adult spleen was implanted on the 8th, 9th, 11th and 13th day of incubation. Implantation on the 15th day of incubation produced only a slight increase in host spleen weight. The second embryonic spleen graft, on the other hand, was stimulated to grow by the adult donor cells, probably because it was well incorporated and contained a sufficient number of viable cells.

These evidences suggest strongly that the host spleen enlargement involves in part a stimulation of the host cells by the adult donor cells. From the work of Till *et al.* (1964) we must consider, in addition, the possibility of host "stem" cell as well as donor "stem" cell proliferation. The nature of the donor host cell interaction or the control mechanisms which could act to alter the activity of the stem cells remains to be explored.

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Summary

1. Adult chicken spleen from a highly inbred line (Line 7) was implanted on the chorioallantoic membrane of embryos from the same line. When the host spleen was transferred to the chorioallantoic membrane from a different strain, a

striking enlargement was obtained, which suggested that adult donor cells were transferred from the first host.

2. When the first host was derived from a different line, and the host spleen was then transferred to another host of the same line as the adult donor, a striking enlargement of the host spleen was observed. This suggests that both adult and host cells were transferred to the second host and also that proliferation of the host embryonic component may have occurred.

3. The splenomegaly phenomenon was inhibited by the intravenous injection of 5-fluorouracil following the implantation of adult chicken spleen. However, the unenlarged host spleen, when transferred to another host embryo, was able to elicit splenomegaly. This reveals the presence of viable and competent donor cells in the treated host spleen.

4. When a fresh piece of 17-day-old chick embryo spleen was added to the chorioallantoic membrane of an embryo which had previously received a graft of adult spleen and an injection of 5-fluorouracil, a significant enlargement of the second embryonic spleen graft was observed.

5. These observations suggest that the splenomegaly reaction is due in part to the proliferation of host embryo cells following stimulation by the adult donor cells.

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